Chapter 3

Oleic acid loading does not add to the nephrotoxic effect of albumin in an amphibian and chronic rat model of kidney injury

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ABSTRACT

Background
In proteinuric conditions, ultrafiltrated albumin can induce an inflammatory and fibrotic response in proximal tubular cells. Unclear is whether albumin per se or compounds bound to albumin are nephrotoxic. Some studies support the toxicity of albumin-bound fatty acids, however they compared untreated, fatty acid containing, albumin and delipidated albumin. To prevent confounding by the delipidation procedure, we compared delipidated albumin and oleic acid-loaded delipidated albumin in 2 models: the classical rat protein-overload and the Axolotl. The latter has an amphibian kidney with a subset of nephrons that drain the peritoneal cavity, so that i.p. injection of albumin selectively targets to open but not to closed nephrons and was used to prevent removal of fatty acids from albumin in the circulation.

Methods
Protein-overload was induced in Wistar rats (groups n = 8, for 12 wks) and Axolotl (groups n = 10; for 10 days) by daily i.p. injections of 1 g (rat) or 50 mg (Axolotl) of fatty acid-free bovine serum albumin (BSA), fatty acid-free BSA with addition of six molecules oleic acid (OA-BSA), or saline (SAL).

Results
After 12 wks, proteinuria and SBP were increased in BSA and OA-BSA rats compared to saline-injected controls ($P < 0.05$), but oleic acid loading had no additional effect compared to albumin alone. This was also true for glomerular and interstitial inflammation, fibrotic changes, and focal glomerulosclerosis (OA-BSA vs BSA, all P = ns). Axolotls injected with oleic acid-loaded albumin showed comparable protein storage in tubular epithelial cells, tubular dilatation and peritubular fibrosis around tubules draining the peritoneal cavity compared with Axolotls injected with albumin alone. This was also true for TGF-ß (inflammation marker) and interstitial collagen I (fibrosis marker) (OA-BSA vs BSA, all P = ns).

Conclusions
In the Axolotl and chronic rat model, oleic acid loading of albumin did not aggravate renal damage compared to albumin alone. Although *in vitro* studies clearly show induction of changes in cultured tubular epithelial cells exposed to albumin-bound fatty acids that are consistent with a role in induction of tubulointerstitial disease, our experiments suggest that currently available models for demonstrating such an effect *in vivo* are insufficient.

INTRODUCTION

In proteinuric conditions, ultrafiltrated proteins reflect the degree of glomerular damage and play an active role in the pathogenesis of chronic tubulointerstitial damage [1,2]. Albumin is the major protein in the ultrafiltrate and nephrotic urine. High *in vivo* and *in vitro* exposure of proximal tubular cells (PTC) to albumin has been shown to induce a pro-inflammatory and pro-fibrotic response [3-5]. Without specific treatment, albumin isolated from plasma always carries other molecules, including fatty acids (FA) [6]. It has been argued that not albumin per se, but rather the FA that are bound to albumin, are toxic to PTC. Macrophage chemotactic activity and apoptosis were increased in cultured PTC in response to exposure to untreated albumin that contains FA compared to delipidated albumin that does not contain FA [7,8]. Untreated FA-containing albumin induced more tubulointerstitial inflammation and glomerular injury in animal protein-overload models than delipidated FA-free albumin [7,9,10]. However, the delipidation procedure may remove other substances besides fatty acids, or modify chemical reactivity or structure of albumin. Therefore, it remains unsure whether the observed effects are due to fatty acids or rather the procedure of delipidation itself. Moreover, in rodent protein-overload studies the albumin-bound FA may never appear in the glomerular ultrafiltrate, because in the circulation FA are loosely bound to albumin, with a t½ in the order of milliseconds [11]. Once FA are not bound to albumin, they tend to be taken up and oxidized in tissues, rather than to stay in the circulation [6,12,13]. Injection doses of palmitate have been shown to almost completely disappear from the circulation within minutes after intravenous injection [6].

We aimed to prevent occurrence of potential confounding by the delipidation procedure by comparing delipidated albumin with delipidated albumin that was subsequently loaded with FA, rather than regular albumin with delipidated albumin. We selectively loaded delipidated albumin with oleic acid because this is the most abundant FA present in human serum [11]. Moreover, oleic acid has been shown to induce fibronectin [14] and ROS production *in vitro* [15]. To this end, we studied the chronic effects of FA-loaded delipidated albumin versus delipidated albumin on renal damage in the classical rat protein-overload model. We also aimed to prevent occurrence of removal of FA during passage of the complex through the circulation, by use of the protein-overload model with FA loaded delipidated albumin versus delipidated albumin in Axolotls (Ambystoma Mexicanum) rather than rats. This amphibian kidney has a unique anatomy with closed and open nephrons; the latter drain the peritoneal cavity via a nephrostome. Injection of proteins into the peritoneal cavity will cause exposure of PTC of nephrons with nephrostomes without passage of albumin through the circulation [16].

METHODS

Albumin solutions for protein-overload (rat and Axolotl)

Fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, Zwijndrecht, Netherlands, catalog no. A-3803) was dissolved in phosphate buffered saline (PBS, pH 7.2) to a solution of 30%. BSA was complexed with oleic acid (OA) (Sigma-Aldrich, Zwijndrecht, Netherlands, catalog no. O-1008) as previously described, resulting in oleic acid-loaded

albumin (OA-BSA) with an OA:BSA molar ratio of 5.2:1 [17]. Low endotoxin levels of the solutions were previously confirmed in our laboratory [17].

Protein-overload in rat

Protein-overload nephropathy was induced in adult male Wistar rats (Harlan, Horst, Netherlands), weighing 220.7 ± 8.6 g (mean ± SD). Rats were housed in a light- and temperature-controlled environment, and had free access to water and standard rat chow. All rats received intraperitoneal injections six times a week for twelve consecutive weeks under isoflurane anesthesia. Rats were injected with either 3.4 ml of saline (SAL, *n* = 8), 1 g of fatty acid-free BSA (BSA, *n* = 8) or with oleic acid-loaded BSA (OA-BSA, *n* = 8). All procedures were approved by the Committee for Animals Experiments of the University of Groningen and the Principles of Laboratory Animal Care (NIH publication no. 85–23) were followed. Body weight was measured weekly. Urine was collected every 4 weeks during a 24-h stay in metabolic cages with access to drinking water only. Tail vein blood samples were taken every 4 weeks under anesthesia. Systolic blood pressure (SBP) was measured in conscious rats by the tail-cuff method. After twelve weeks, the rats were anesthetized, a blood sample was taken by cannulation of the aorta, and the kidneys were perfused with saline. A coronal tissue slice was snap-frozen in isopentane and stored at -80°C. Another coronal tissue slice was fixed in 4% paraformaldehyde and processed for paraffin embedding.

Proteinuria was measured colorimetrically with the Pyrogallol-Red Molybdate method. Plasma and urine creatinine levels were determined by the Jaffé method (Merck Mega, Darmstadt, Germany).

Immunohistochemistry

Paraffin sections (4 µm) were stained with periodic acid-Schiff (PAS) to evaluate focal glomerulosclerosis (FGS). Immunostaining was performed on paraffin sections for α-smooth muscle actin (α-SMA, clone 1A4, dilution 1:15,000, Sigma, St. Louis, MO, USA) and macrophages (ED1, dilution 1:1000, Serotec, Oxford, UK). Deparaffinized sections were subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer (pH 9.0) at 80ºC. Endogenous peroxidase was blocked with 0.075% H_2O_2 in PBS (30 min). Primary antibodies diluted in 1% BSA/PBS, were incubated for 60 min at room temperature. Binding was detected using sequential incubations (30 min) with appropriate peroxidase (PO)-labeled secondary antibodies (DakoCytomation), diluted in PBS with 1% BSA and 1% human serum. Peroxidase activity was developed using 3,3'-diaminobenzidine tetrachloride (DAB, 10 min). Sections were counterstained with haematoxylin. Appropriate isotype and PBS controls were consistently negative.

Focal glomerulosclerosis (FGS) was semi-quantitatively scored (scale 0-4) in PAS-stained sections and expressed as the mean score of 50 glomeruli per kidney. FGS was scored positive when mesangial matrix expansion and adhesion of the visceral epithelium to Bowman's capsule were simultaneously present. A score of 1 was given when 25% of the glomerulus was involved, 2 for 50%, 3 for 75%, and 4 for 100%. Computerized morphometry was used to measure glomerular α-smooth muscle actin (α-SMA) (50

glomeruli), and interstitial α-SMA (30 randomly selected cortical fields; vessels and glomeruli were excluded). The total staining area was divided by the total surface area and expressed as percentage. For glomerular macrophages (MØ), the number of positive cells per glomerulus (n=50) was counted. The number of interstitial macrophages was counted per interstitial field (average of thirty fields per kidney) using a 10x10 grid at a magnification of 200x (with a total area of 0.25 mm^2 per interstitial field); vessels and glomeruli were excluded from measurements. Interstitial macrophages are presented as number per interstitial field.

Protein-overload in Axolotl

Eighteen-month-old neotenic Axolotls of both sexes, weighing between 80 and 120 g, were derived from the Axolotl Colony of the University of Indiana. Animals were held in tanks of aerated tap water at a constant temperature of 18°C with a 12-hour light (06.00 to 18.00 hours) and 12-hour dark cycle (18.00 to 06.00 hours). They were fed with pellets of fish food. One week prior to the study, animals were randomly allotted to three groups of 10 animals each. The daily BSA dose was chosen based on work of Gross et al [16] who did not find tubulointerstitial disease after intraperitoneal injection of glycated human albumin in a concentration of 4.5 g/dL for 6 days (the daily injected amount of albumin was ~22.5 mg) and work of Hein et al [18] who found significant induction of tubulointerstitial disease after intraperitoneal injection of 0.5 mL of human albumin (probably corresponding to ~20 mg of albumin) for 6 consecutive days and. To prevent potential underdosing, the animals received daily intraperitoneal injections for 10 consecutive days of either saline (SAL), 50 mg fatty acid-free BSA (BSA, *n* = 10) or oleic acid-loaded BSA (OA-BSA, *n* = 10). At the end of the observation period, a blood sample was obtained under general anesthesia (3-aminobenzotic acid ethyl ester, A-5040; 10 g/L water in the tank; Sigma) and retrograde perfusion was performed via the main heart ventricle. For immunohistochemistry, animals were perfused with ice-cold isotonic saline. The kidneys were then excised. One part was snap-frozen and the other part was fixed with 4% formalin.

Light Microscopy

Paraffin sections (4 µm) were stained with a connective tissue stain (Ladewig stain) [19] and examined using light microscopy at a magnification of 100x. The tubulointerstitial changes were quantified by a "blinded" examiner (MvT), who was unaware of the assignment to the treatment groups, using a score system by comparison with a set of photos with standardized lesions, i.e. tubular dilatation, protein droplet content of tubular epithelial cells, and peritubular interstitial fibrosis around protein storing tubules. For protein droplet content scores were assigned for the quantity of the tubules containing protein droplets (0-4; 0 is 0; 1 is <25% of the tubules containing droplets; 2 is 25-50%; 3 is 50-75%; 4 is >75% of the tubules containing protein droplets) and for the quality of the droplets (0-3) according to the size of the droplets. The size of the droplets was scored semi-quantitative on a scale of 1 to 3. Small droplets (~0.5 µm) is 1; moderate droplets (~ 2 µm) is 2; large droplets (~ 5 µm) is 3. The ultimate score for protein droplet content was given by multiplying the quantity and quality score (range 0-12). For peritubular

interstitial fibrosis the following scores were assigned of: 0 is no change; 1 is minimal change; 2 is moderate change; 3 is marked change; 4 is very pronounced change. For tubular dilatation a score of 0 corresponded to an average diameter of 50 µm, 1 to 100 µm, 2 to 150 µm, and 4 to 250 µm or more.

Immunohistochemistry

Immunostaining was performed as described above on paraffin sections for TGF-ß (TGF-ß1, SC-146, dilution 1:100, Santa Cruz Biotechnology, Santa Cruz, USA) and anti-collagen I (rabbit anti rat collagen I, AB 755, Lot 131 DDM, dilution 1:200, Chemicon International Inc. Temecula, CA, USA).

Glomerular, tubular and interstitial structures were assessed using a score system. Tubular epithelial cells and interstitial cells were separately quantified, evaluating the area and the intensity of staining. The scores were defined as: 0 is no staining, 1 is minimal staining, 2 is moderate staining, 3 is marked staining, 4 is very pronounced staining.

Statistical analysis

Data are presented as median and range. Statistical analyses were performed with SPSS Version 12.0 (SPSS Inc., Chicago, IL, USA). Differences between groups were detected with the non-parametric Kruskall-Wallis test. A two-sided P-value <0.05 indicated statistical significance.

RESULTS

Rat: Clinicopathological parameters and renal damage

Clinicopathological parameters are shown in Table 1. At week 12 bodyweight was the same in all treatment groups. The BSA and OA-BSA overloaded rats did not differ in urinary protein excretion, creatinine clearance and SBP, but these parameters were in both groups – as expected – significantly increased when compared to saline-injected controls ($P < 0.001$ for urinary protein excretion and creatinine clearance; $P < 0.05$ for SBP). The course of development of proteinuria in the different groups is shown in Figure 1. At all timepoints there was an insignificant trend towards less proteinuria in the OA-BSA overloaded rats compared with the BSA overloaded rats.

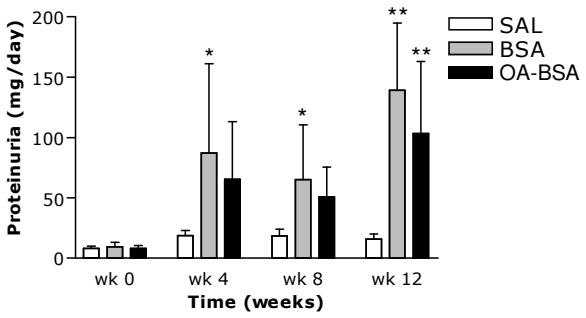

Figure 1. Proteinuria in the three different treatment groups: saline-injected control rats (SAL), delipidated albumin (BSA), and oleic acid-loaded delipidated albumin (OA-BSA). * P<0.05 and ** P<0.01 vs SAL.

Table 1. Clinicopathological parameters rat (week 12) and Axolotl model (day 10)

RAT	SAL	BSA	OA-BSA
Bodyweight (g)	440.8 ± 31.9	438.3 ± 47.3	426.7 ± 37.4
Proteinuria (mg/d)	15.7 ± 4.3	139.0 ± 55.6 #	103.3 ± 59.6 #
SBP (mm Hg)	126.6 ± 5.9	137.1 ± 6.3 *	141.1 ± 7.3 *
CrCl (ml/min/100g bodyweight)	0.751 ± 0.087	1.098 ± 0.194 #	1.103 ± 0.187 #
AXOLOTL			
Bodyweight (g)	113 ± 15	107 ± 13	110 ± 12
Kidney weight (mg)	103.6 ± 48.2	87.1 ± 18.9	108.3 ± 47.4
Heart weight (mg)	50.2 ± 21.6	39.0 ± 9.0	56.8 ± 21.2

SAL = saline; BSA = BSA-injected; OA-BSA = oleic acid-loaded BSA-injected; SBP = systolic blood pressure; CrCl = creatinine clearance. * P<0.05 vs SAL; # P<0.001 vs SAL.

The scores of histological parameters are given in Figure 2. Representative photographs of histological sections are shown in Figure 3. Between BSA and OA-BSA groups there were no significant differences in glomerular (mean ± SD 3.9 ± 1.1 vs. 4.4 ± 1.3 macrophages per glomerulus resp., P = 0.46) and interstitial macrophages (87.4 ± 28.6 vs 84.7 ± 25.8 macrophages per interstitial field, resp., P = 0.60). Also, glomerular (1.2 ± 0.6 vs. 1.2 ± 0.8 % per glomerulus, resp., P = 0.92) and interstitial (2.0 ± 1.0 and 1.7 ± 0.4 % per interstitial field, resp., P = 0.83) α-SMA expression did not significantly differ between BSA and OA-BSA overloaded rats. The same was true for focal glomerulosclerosis (FGS) (0.10 ± 0.08 vs. 0.07 ± 0.06 arbitrary units, resp., P = 0.26). Compared to the saline-injected control group, the extent of glomerular macrophages, interstitial α-SMA expression and FGS was significantly increased in both BSA and OA-BSA overloaded rats (resp. values were 2.1 ± 0.6, 0.6 ± 0.2, and 0.0 ± 0.0 in control rats, P < 0.01 for all six comparisons). Scores for interstitial macrophages and glomerular α-SMA expression in BSA and OA-BSA were not significantly different from saline-injected controls (resp. values were 64.5 ± 32.3 and 0.9 ± 0.6 in control rats, P-values ranged between 0.15 and 0.25 for the four comparisons).

Axolotl: Clinicopathological parameters and renal damage

Clinicopathological parameters are shown in Table 1. At day 10 bodyweight, kidney weight and heart weight were the same in all treatment groups. The scores of histological parameters are given in Figure 3. Representative photographs of histological sections are shown in Figure 4. In the BSA and OA-BSA injected animals, tubular protein storage was only noted in tubules that drained the peritoneal cavity via a nephrostome (Figure 4A), confirming the selective uptake of proteins in PTC in open, but not in closed, nephrons [16]. Furthermore, tubular dilatation and peritubular fibrosis was found in areas in close vicinity to protein-loaded tubules (Figure 4B). Between BSA and OA-BSA groups there were no significant differences in tubular protein storage (4.6 ± 3.9 vs. 5.9 ± 3.4 arbitrary units, resp., P = 0.27), tubular dilatation (2.3 ± 1.0 vs. 2.1 ± 1.1 arbitrary units, resp., P = 0.63) and fibrosis (1.6 ± 0.4 vs. 1.3 ± 0.7 arbitrary units, resp., P = 0.28) (determined on a connective tissue Ladewig stain). Compared to the saline-injected control group the extend of tubular protein storage and tubular dilatation was significantly increased in both BSA and OA-BSA overloaded Axolotls (resp. values were 1.5 ± 2.4 and 0.7 ± 0.9 in controls, both P < 0.05). Fibrosis was significantly increased in BSA (P < 0.001) and non-

significantly in OA-BSA (P = 0.07) overloaded Axolotls (value was 0.8 ± 0.6 in controls). Immunohistochemical stainings confirmed these results. TGF-ß staining, used as inflammatory marker, was found in epithelial cells (Figure 5C-D). Collagen I staining, used as a marker of fibrosis, was found in epithelial and interstitial cells (Figure 5E-F). Between BSA and OA-BSA groups there were no significant differences in TGF-ß (3.2 ± 0.6 vs 2.8 ± 0.8 arbitrary units, resp., P = 0.49) and collagen I (7.9 ± 7.3 vs 4.6 ± 3.3 arbitrary units, resp., P = 0.39) staining. TGF-ß and collagen I staining in both BSA and OA-BSA groups were significantly increased when compared to saline-injected controls (0.9 ± 0.5 and 1.6 ± 2.4 in controls, resp., P < 0.05 for all four comparisons).

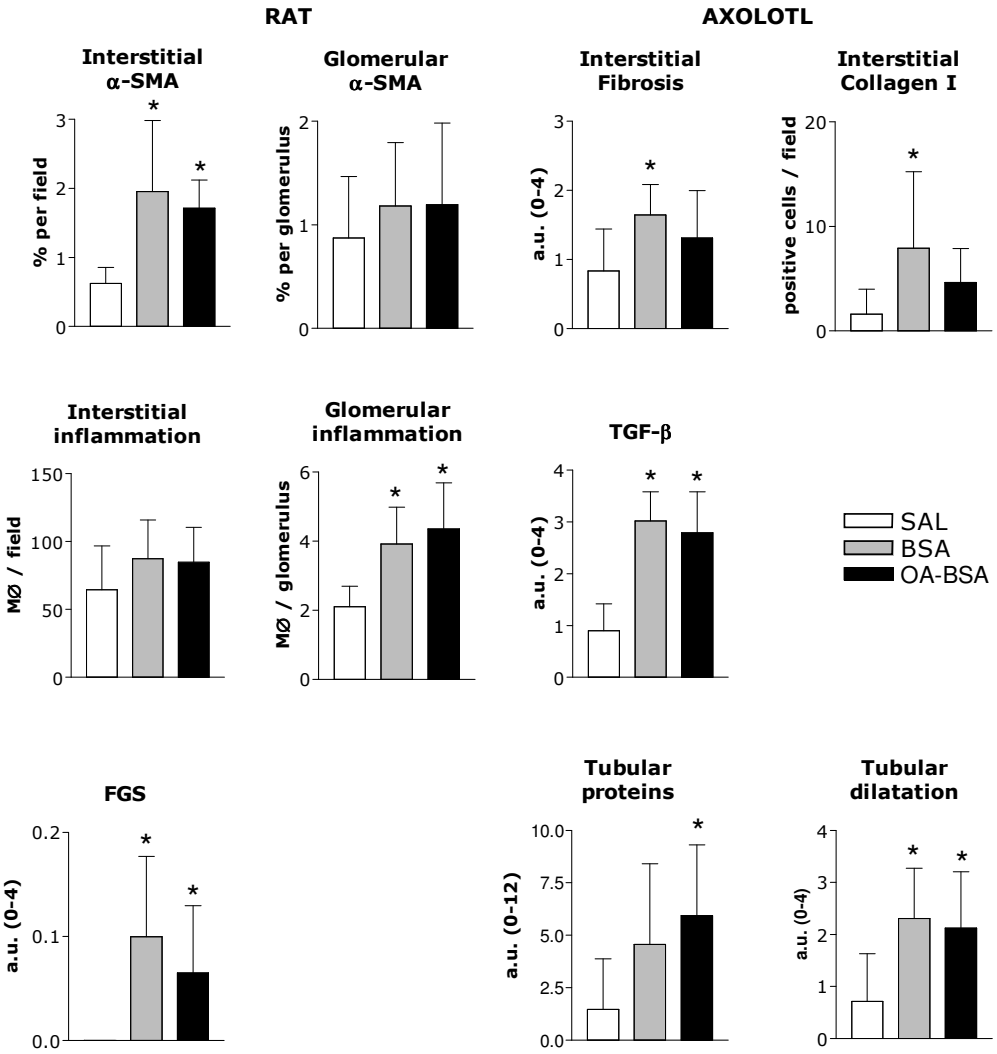

Figure 2 (on page 44)

Scores of renal damage parameters in rat (left) and Axolotl (right) of the three different treatment groups: saline-injected controls (SAL), animals injected with delipidated albumin (BSA) and with oleic acid-loaded delipidated albumin (OA-BSA). * $P < 0.05$ vs SAL.

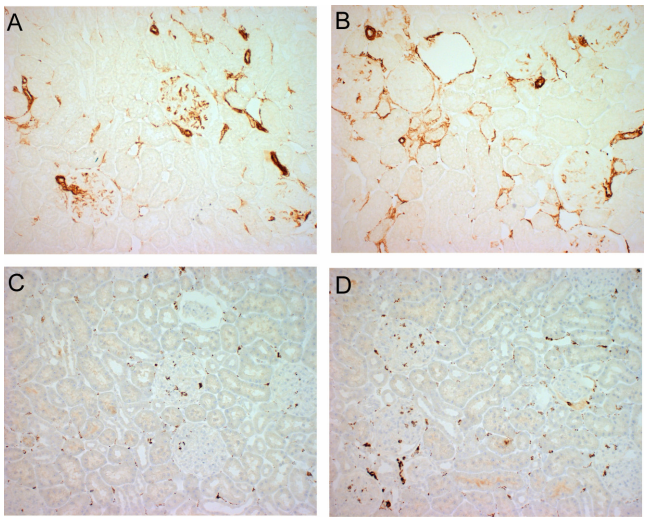

Figure 3. Protein-overloading in rat.

A) In saline-injected control kidneys interstitial α-SMA expression is not very abundant, whereas glomerular α-SMA expression is more pronounced. B) In protein-overloaded kidneys (both BSA and OA-BSA) increased interstitial α-SMA expression was found, while glomerular α-SMA expression was not changed. C) In saline-injected control kidneys little interstitial and glomerular macrophages were found. D) In protein-overloaded kidneys (both BSA and OA-BSA) the number of interstitial macrophages was not changed, while an increased number of glomerular macrophages was found. An immunohistochemical stain is shown. Magnifications are 200×.

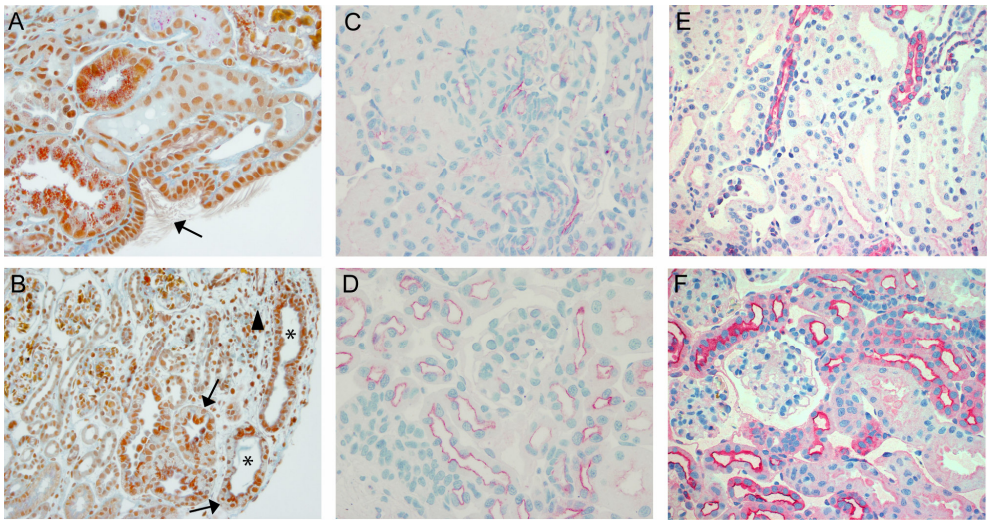

Figure 4. Protein-overloading in Axolotl.

A) Ciliated funnel on the peritoneal surface of an Axolotl kidney. Cilia are present on the peritoneal surface and in the funnel (arrow). After protein loading, protein droplets are found in tubules that drain the peritoneal cavity, and not in other tubules. B) After protein loading, tubular epithelial cells in tubules that drain the peritoneal cavity were swollen and filled with protein droplets (arrows). Sometimes tubular dilatation was noted in tubules with protein droplets (stars). Accumulation of interstitial tissue around tubules with protein droplets was also seen (arrowheads). C) In saline-injected control kidneys virtually no TGF-ß expression was found. D) In protein-overloaded kidneys increased tubular TGF-ß expression was found. E) In saline-injected control kidneys little tubular collagen I expression was found. F) In protein-overloaded kidneys increased collagen I expression was found. In A and B a Ladewig stain is shown, while C-F show an immunohistochemical stain. Magnifications are 100× (A-B) and 200× (C-F).

DISCUSSION

In the current study we show that oleic acid loading of albumin has no additional effect on renal damage when compared to albumin alone. In both the Axolotl and the chronic rat model, proteinuria, renal inflammation and fibrotic changes were comparable in the groups injected with delipidated oleic acid-loaded albumin and delipidated albumin. These results suggest that the delipidation procedure itself may have been responsible for observations of less renal injury after injection of delipidated albumin versus regular albumin in previous studies.

Other rodent protein-overload studies have provided evidence for the detrimental effects of albumin-bound FA on renal structure [9,10]. The experimental set-up in these studies was different from our set-up, for example Thomas et al [10] did the experiments in Lewis rats and gave twice as much albumin for 7 days. This makes a direct comparison with our study difficult. The most prominent difference with our study was however, that in the above described experiments a comparison was made between untreated albumin – that

always carries other molecules, including fatty acids – and delipidated albumin. In our study we compared delipidated albumin with delipidated albumin that was selectively loaded with oleic acid. Delipidated oleic acid-loaded albumin has been shown to induce ROS production *in vitro* [15]. In addition, in a short term, i.e. 3 weeks, rat protein-overload model we have previously shown that oleic acid-loaded albumin induced more renal inflammation and prefibrotic changes than delipidated albumin, although no effect was seen on the level of proteinuria [17]. Surprisingly, in the current long-term, i.e. 12 weeks, protein-overload model renal inflammation and fibrosis, as well as proteinuria, were similar in BSA and OA-BSA overloaded rats. The discrepancy between short- and long-term effects of OA-BSA may have different explanations. The results of the short term study can be a chance hit. The same may of course apply to the results of this study, however, the results of our current long-term protein-overload study did not approach statistical significance, and if trends existed, they were more in the direction of a protective effect of oleic acid than a harmful effect. It is also possible that in the current long-term protein-overload model the additional effects of oleic acid on renal damage that were seen in the short-term model, were overruled by the deleterious effects of albumin and the vicious circle of tubulointerstitial damage and inflammation that was consequently induced. If this is true, the contribution of fatty acids in the long term, i.e. the clinically more relevant time period, seems neglectable. Obviously, before a definite conclusion about absence of an effect of albumin loaded with oleic acid on induction of tubulointerstitial damage can be drawn, our findings require independent confirmation by other investigators.

Another point that needs to be addressed in previous rodent protein-overload studies is the possibility that the albumin-bound FA may never appear in the glomerular ultrafiltrate. Although albumin is the major carrier of FA in the circulation, FA are only loosely bound to albumin [11]. FA that are not bound to albumin are rapidly taken up by the tissues and consequently disappear from the circulation [6]. To overcome the removal of FA from albumin during passage through the circulation, we therefore evaluated the direct effects of albumin-bound FAs on PTC in the Axolotl. This amphibian kidney has a unique anatomy in which ciliated peritoneal funnels, nephrostomes, connect the proximal tubule to the peritoneal cavity. Due to these nephrostomes the Axolotl kidney possesses 2 different sets of nephrons: "normal" closed nephrons, in which glomerulus and tubule form a closed unit, and open nephrons that connect via a nephrostome to the peritoneal cavity. Hein et al [18,20] and Gross et al [16] have previously shown that the Axolotl kidney can serve as model to study tubulointerstitial activation and induction of interstitial fibrosis by protein loading. Injection of protein into the peritoneal cavity fails to expose closed nephrons to a protein load, but causes selective uptake and storage of proteins in tubular epithelial cells of nephrons with nephrostomes [16]. We confirmed this selective uptake and storage of proteins in PTC belonging to open nephrons, but we furthermore showed that oleic acid loading of albumin did not aggravate renal damage compared to albumin alone. Although Gross et al [16] could not demonstrate an effect of glycated human albumin (~22.5 mg injected daily for 6 days) compared to saline-injected controls, our results confirm preliminary results of another study by Gross et al [21] in which it was shown that Axolotls

overloaded with lipid poor and lipid rich albumin - for 10 days with 25 mg of albumin - had equal amounts of tubular protein storage and peritubular fibrosis. However, like in rats in the circulation it can not be excluded that the fatty acids dissociate from albumin in the peritoneal cavity in the Axolotl model.

Also, in cultured proximal tubular cells the effects of delipidated albumin in comparison with untreated, lipidated albumin are not conclusive. Although several studies demonstrated that FA carried on albumin aggravated the deleterious effects on PTC compared to delipidated albumin [7,8,14], other studies have failed to show this. PTC stimulated with untreated lipidated albumin and delipidated albumin showed equal levels of proliferation and hypertrophy and synthesized equal levels of endothelin-1 and MCP-1 [22-24]. The initiation of inflammatory and fibrotic cascades is likely to be dependent on endocytosis of albumin. It appears that renal tubular cell lines with low rates of endocytic uptake are resistant to the profibrotic effects of albumin [25].

Which proteins play the most predominant role in the activation of PTC towards a more pro-inflammatory and pro-fibrotic state is still unanswered. In addition to albumin, and albumin-bound compounds, several other filtered proteins have been suggested to induce changes in PTC, including proteins of the complement system, immunoglobulins, and growth factors. To what extent these proteins contribute to inflammatory and fibrotic effects is unclear, although some studies indicate differential and protein-specific effects [22,26,27].

How can proteins, or more specific albumin, or albumin-bound FA exert an effect on PTC? Although the exact pathway has not been established, evidence suggests that the process involves the initial endocytic uptake of albumin. In humans and rodents, albumin in the tubular lumen binds to the receptors megalin and cubulin in the luminal surface of PTC [1,28]. After binding, it is then internalised while bound to these receptors and subsequently degraded to it constitutive amino acids. Additional uptake mechanisms of albumin have been suggested, including endocytosis via the epidermal growth factor (EGF) receptor [29] and reabsorption via a high-capacity transcellular pathway that transfers intact albumin into the peritubular blood supply [30]. However, to what extent these different pathways contribute is unclear. A key consequence of albumin overload is the production of the proinflammatory cytokine MCP-1 [24], the profibrotic cytokine TGF-ß [31] and collagen [25] and these are dependent on the endocytosis of albumin. However, a recent study has shown that induction of TGF-ß by exposure to albumin does not depend on the endocytosis of albumin [32]. Whether the above mentioned pathways are also present in the Axolotl kidney is unclear, but as is shown that proteins accumulate in PTC, it seems very likely that at least some of them are present. Nevertheless, we did not observe differences in response to tubular loading with delipidated albumin and oleic acid-loaded albumin.

In conclusion, we studied the effects of oleic acid-complexed albumin on the induction of renal damage. To overcome potential confounding by the delipidation procedure we compared delipidated albumin with oleic acid-loaded albumin. We compared the effects in the classical rat protein-overload model, in which albumin reaches the kidney via the circulation, and in the Axolotl in which albumin is directly delivered to the tubules and

thereby omits the possible disturbing effects of the circulation on albumin. In both models we demonstrated that oleic acid loading of albumin has no additional effect on renal damage when compared to albumin alone. Although *in vitro* studies clearly show induction of changes in cultured tubular epithelial cells exposed to albumin-bound fatty acids that are consistent with a role in induction of tubulointerstitial disease, our experiments suggest that currently available models for demonstrating such an effect *in vivo* are insufficient. One reason may be that fatty acids attached to albumin never reach the tubular lumen, even in the Axolotl model, in which there are tubules that directly drain the peritoneal cavity.

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